CONFIDENTIAL – NEW PROTOCOL SUBMISSION – This information must remain confidential until the protocol is approved and claims have been registered using the approved protocols.

Protocol

RB PROTOCOL TO ASSESS REDUCTION IN VIRAL CONTAMINATION IN INDOOR AIR

Test Microorganism(s):

Coliphage MS-2 (ATCC 15597-B1) with host *Escherichia coli* (ATCC 15597)
Cystovirus/Phi6 (ATCC 21781-B1) with host *Pseudomonas syringae* (ATCC 19310)
(*Additional viruses may be listed.)*

Performing Laboratory:

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Sponsor

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Test Guideline

EPA OCSPP 810.2500

Protocol Identification No.

To be identified

May 18, 2021

RB PROTOCOL TO ASSESS REDUCTION IN VIRAL CONTAMINATION IN DOOR AIR

1.0 Objective

1.1 This document describes assessment of a test substance for a temporary reduction in the number of viable bacteriophages / viruses in indoor air (Ijaz et al 2016; Kashkooli et al. 2019; Sattar et al. 2016; Zargar et al. 2016; 2019). Bacteriophages as surrogates for vertebrate viruses (enveloped and non-enveloped) have been used for aerobiological investigations including assessment of air decontamination technologies (Duchaine, 2016; Fedorenko et al, 2020; Turgeon et al., 2014; Sattar et al. unpublished data), Biosafety concerns and experimental challenges posed by working with the influenza virus and coronaviruses such as SARS-CoV-2, and noroviruses, this protocol employed the enveloped bacteriophage Phi6, used as a surrogate for the influenza virus (Adcock et al., 2009; Turgeon et al., 2014) and SARS coronavirus (Casanova et al., 2016), and MS2 for noroviruses (Dubuis et al., 2020). Therefore, using bacteriophages as the surrogates of vertebrate viruses can be an alternative to overcome the difficulties, expense, and most importantly biosafety aspects of working with vertebrate viruses.

2.0 Good Laboratory Practice

This study will be conducted in accordance with EPA Good Laboratory Practice (GLP) regulations (40 CFR Part 160). If necessary, an external Quality Assurance Unit may be provided for this study. The following exceptions to EPA Good Laboratory Practice may be noted in the final study report:

For the studies not performed by or under the direction of the Test Facility, including test substance characterization (40 CFR Part 160.105), a Certificate of Analysis for characterization will be appended to the study report.

The computer equipment used in the BSL-2 to capture the room temperature and relative humidity (RH) readings may not be handled in accordance with 40 CFR Part 160. The captured data will be printed following each experiment, initialed and dated, and archived in the study file for permanent storage.

The photography equipment and retention of the electronic form of photos taken during study conduct may not be handled in accordance with 40 CFR Part 160. The photos will be printed, initialed and dated, and archived in the study file for permanent storage.

An external Quality Assurance Unit (QAU) may be engaged to review and certify the quality of the data recorded for the study.

3.0 A glossary and list of abbreviations may be found in Appendix 3

4.0 Purpose

4.1 The purpose of this study is to evaluate the ability of a test substance to provide a temporary reduction in the number of bacteriophages/viruses in an aerobiology chamber to support air treatment labeling claims.

5.0 Justification for Selection of the Test System

5.1 The study design and test system comply with the U.S. Environmental Protection Agency (EPA) OCSPP 810.2500 Air Sanitizers – Efficacy Data Recommendations (December 2012) with the following exceptions:

3.1.2 An allowance has been inserted for selection of airborne viruses to support additional claims (e.g. SARS/CoV-2/human coronavirus 229E, influenza virus, human norovirus/murine norovirus*)* as desired.

6.0 Scope

6.1 This document outlines the procedure to assess the ability of air decontaminating agents to inactivate representative viral pathogens in indoor air.

6.2 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the procedures described here must be documented and justified.

7.0 Experimental Dates

7.1 The proposed experimental start date is {*insert date*}.

7.2 The proposed experimental termination date is {*insert date*}.

8.0 Test Substance Characterization

8.1 In accordance with 40 CFR Part 160.105, test substance characterization as to identity, strength, purity, solubility, and composition, as applicable, will be documented before its use in this study. The stability, if appropriate, will be determined prior to or concurrently with this study.

8.2 The sponsor will report if the characterization and stability studies have been performed under GLP by filling up the Test Substance Characterization GLP Compliance Assessment form. Which will be appended to the study report. Characterization and stability studies which are not performed under the GLP will be exempted in GLP compliance statement.

8.3 A Certificate of Analysis (CoA) summarizing the chemical characterization will be appended to the study report. Characterization and stability studies not available at the performing laboratory will be exempted in GLP compliance statement.

8.4 The test substance will be prepared in accordance with EPA Lower Certified Limit Guidance found in 810.2000 (February 2018).

8.5 See Section 23.0 for chemical analysis conducting during testing.

9.0 Test Substance

9.1 Test Substance Name: {Insert name}

9.2 Test Substance Batch Number: {*For testing of viruses or phages, two batches will be used*. The virus representing the worst case structure per EPA 810.2200 will be evaluated using batches meeting the EPA Lower Certified Limit guidance provided in 810.2000 (2018) and all other viruses may be tested at or below the nominal certified limit.}

9.3 Test Substance Storage Conditions: {Insert conditions}

9.4 Test Substance Preparation: {*Insert preparation instructions including required dilutions and diluents*}

9.4.1 For concentrates, the diluent used in testing and described in labeling will be consistent with EPA guidance in 810.2000 (2018).

9.5 Test Substance Application Method: {*Insert air treatment instructions. The labeled use instructions will be based upon the technique used during testing (e.g. For an aerosol, spray can continuously for "x" seconds in center of the room aiming at the ceiling to treat the air.)*}

9.6 Test Substance Exposure Time(s): {Insert exposure time(s) (e.g., 5, 10, 60 mins)}: The

exposure or dwell time of the released chemical in the air will be measured and documented.

9.7 Test Substance Exposure Air Temperature: {*Insert temp. with range* (e.g., 20-25°C) }

9.8 Test Substance Exposure Relatively Humidity (RH) {*Insert RH with range* (e.g., 50±5%)}

9.9 Test Substance Retention is the responsibility of the Sponsor. All unused test substance will be discarded following study completion unless otherwise indicated by the Sponsor.

9.10 See Section 23.0 for chemical analysis conducting during testing.

10.0 Labware:

10.1 All items are to be sterile and disposable; they are available from any supplier of scientific labware.

10.2 Micropipettes with appropriate tips to accurately deliver 20, 100 or 1000 µL volumes

10.3 Screw cap tubes – 2 mL

10.4 Screw cap tubes - 50 mL

10.5 Screw cap tubes -15 mL

10.6 Petri plates -150 X 25 mm

10.7 Petri plates -100 X 15 mm

10.8 Serological Pipettes – 1, 5, 10 and 25 mL capacity

11.0 Personal protective equipment (PPE; all items listed below are available from suppliers of laboratory safety gear); the requirements for PPE may vary from site to site.

11.1 Safety glasses

11.2 Laboratory gloves

10.0 General Solutions and Reagents

10.1 Deionized distilled water (DDW) or equivalent, for making reagent solutions and media.

10.2 Luria Bertani (LB), to culture the test host bacteria.

10.3 Luria Bertani (LB) agar, to recover the phage from control and test samples and for conducting sterility tests.

10.4 Antifoam A concentrate (Sigma-Aldridge, St. Louis, MO; Cat. # A-5633), is added to the bacteriophage/viral suspension to be nebulized to reduce frothing. This autoclave-sterilizable silicon-based item is commonly used in fermentation systems and is devoid of bactericidal or bacteriostatic activity.

10.5 Earl's balanced salt solution (EBSS)

10.6 Dulbecco's PBS.

10.7 Glycerol

11.0 Soil load (ASTM International 2013; OECD 2013; Springthorpe and Sattar, 2007). The same soil load will be used for all test microorganisms to be nebulized.

11.1 The soil load for incorporation into the bacteriophage / viral suspension to be nebulized consists of a mixture of the following thawed stock solutions in phosphate buffered saline (PBS; pH 7.2±0.2):

11.1.1 0.5 g of yeast extract in 10 mL of PBS.

11.1.2 0.5 g of bovine serum albumin (BSA) in 10 mL of PBS.

11.1.3 0.04 g of bovine mucin in 10 mL of PBS.

11.2 The stock solutions of all three components of the soil load are sterilized by passage through a syringe-mounted (25 mm diameter) polyethersulfone (PES) membrane (0.22 µm pore diam.).

11.3 All three solutions are then aliquoted as 1.5 mL volumes and stored at -20±2°C with a shelf-life of at least one year. For short-term storage, the vials can be kept at 4±2°C for no longer than 90±5 days.

12.0 General equipment

12.1 Air displacement pipettes (Eppendorf or equivalent) with tips to dispense 100 to 1000 µL volumes.

12.2 Analytical balance, to weigh chemicals and to record and standardize inoculum delivery volumes as well as calibration of pipettes.

12.3 Centrifuge to attain speeds of 3,000xg or higher, to allow for the sedimentation of the test host cells for bacteriophages / viruses for concentration or washing, or both.

12.4 Freezer: At -20±2°C is required for the storage of media, reagents and additives.

12.5 Deep freezer: At -70°C or lower to store the stocks of test microorganisms.

12.6 Incubator: To maintain a temperature of 36±1°C for the culture of the test microorganisms and also for sterility testing.

12.7 Biological safety cabinet (BSC), Class II (Type A): Certified; please refer to *Biosafety in Microbiological and Biomedical Laboratories* (CDC 2020) for proper maintenance and operation of this piece of equipment. The performing laboratory must have its own standard operating procedure (SOP) for the certification, maintenance and operation of such devices.

12.8 Refrigerator: at 4±2°C for storage of media, culture plates and reagents.

12.9 Autoclave, to sterilize culture media, reagents and waste.

12.10 Vortex mixer, to homogenize microbial suspensions.

12.11 Balance to weigh the nebulizer before and after spraying of the microbial suspension.

12.12 Waterbath

13.0 Examples of Specialized equipment (More details are given in Appendix 1)

13.1 Slit-to-agar (STA) programmable sampler: For event-related collection of viral aerosols (e.g., Pinpoint Scientific Ltd, 1st Floor, North Road, Bridgend Industrial Estate, Bridgend,CF31 3TP; sales@pinpointscientific.com).

13.2 Six-jet Collison nebulizer: To generate microbial aerosols in the respirable range of 0.5-5.0 µm (e.g., CH Technologies., 778 Carver Ave, Westwood, NJ 07675, www.chtechusa.com); cylinder of extra-dry compressed air with pressure regulator and a back flow preventer.

13.3 Volatile-gas detector with a gas leak probe: To check for any air leaks from the chamber (e.g., Model BT-45; Quantum Instruments, Garden City, NY).

13.4 Air temperature and RH meter: To monitor and record the air temperature and RH in the aerobiology chamber via a wireless data logger (e.g., CAS Data Loggers, 8437 Mayfield Rd., Unit 104 Chesterland, OH 44026).

13.5 Magnehelic: To detect any pressure differential between the inside and outside of the chamber (Figure 1) (ITM instrument Inc. 16975 Leslie St. Newmarket, ON L3Y 9A1).

13.6 A muffin fan to evenly distribute the aerosolized microorganism inside the chamber and to keep them airborne during testing.

13.7 Device for the collection of the test substance from the chamber air

14 **Viruses and their hosts for the testing**.

14.2 Since contamination of stock cultures can negatively impact the test data, it is crucial to abide by the highest standards of GLP during all manipulations and handling of stock and working cultures.

14.3 All manipulations of the test microorganisms must be performed in accordance with the biosafety practices stipulated in the relevant SOPs of the performing laboratory.

Table 1. Examples of Test Bacteriophages / Viruses

Virus (ATCC #)	Host cell & Incubation	Justification
MS-2 (15597-B1)	*Escherichia coli* (15597); 36±1°C	Small-sized (~30 nm), non-enveloped with RNA genome; often used as a surrogate for non-enveloped human pathogenic viruses (e.g., noro- and rhinoviruses)
Phi6 (4352-B1)	*Pseudomonas syringae* (31952); 30±1°C	Medium-sized (~100 nm), enveloped with RNA genome; often used as a surrogate for enveloped human pathogenic viruses (e.g., corona- and influenza viruses)
Human influenzavirus PR8 (VR-95)	MDCK (CCL-34); 36±1°C	Medium-sized (~100 nm), enveloped respiratory virus known to spread via inhalation of contaminated indoor air.
Human coronavirus 229E (VR-740)	MRC-5 (CCL-171); 33±1°C	Medium-sized (~100 nm), enveloped respiratory virus known to spread via inhalation of contaminated indoor air. Frequently used as a surrogate for more pathogenic coronaviruses (e.g., SARS CoV-2) known to spread via inhalation of contaminated indoor air.
Murine norovirus Strain S99	RAW 267.7 (TIB-71); 36±1°C	Small-sized (~30 nm), non-enveloped with RNA genome; often used as a surrogate for human noroviruses known to spread via inhalation of contaminated indoor air.

14.3 Maintenance, passage and storage of test viruses:

14.3.1 Obtain standard strains of the host bacteria (lyophilized), bacteriophages, mammalian viruses and their host cells to be used in the testing from a reputable source such as the American Type Culture Collection (ATCC). Every 18 months (or sooner if the quality of the stock culture is compromised) new stock cultures of the test organisms.

14.3.2 Use the following procedures to initiate and maintain in-house stocks of the cultures.

14.4 **Culture Initiation of host bacteria**

14.4.2 Wipe the outside of the ampule/vial with a towelette prewetted with 70% (v/v) ethanol and open it inside a laminar flow hood.

14.4.3 Resuspend the contents in 1.0 mL of the media recommended by ATCC.

14.4.4 Using a pipettor with a sterile pipette tip place 0.1 mL of the rehydrated suspension into each one of two 10.0 mL tubes containing 5.0 mL of sterile the media recommended by ATCC. Mix well by shaking.

14.4.5 Streak a loopful of the suspension onto two 100 mm diameter agar plates recommended by ATCC. (predried to remove any accumulated water on the surface of the agar) to obtain isolated colonies, and incubate the plates at the required temperature for 18±2 h.

14.4.6 Observe the plates for growth and typical colony morphology of the bacterium. For example, the colonies of *E. coli* should appear round, convex, entire, glossy, creamy-colored colonies ~2 mm in diameter; colonies of *P. syringae* should be mucoid, circular and raised with an undulate margin and a blue-green pigment surrounding the colony with a colony diameter of 3-4 mm.

14.4.7 Prepare a smear from an isolated colony, Gram-stain it and observe the smear microscopically under an oil-immersion objective (1000X) to ascertain that the Gram-reaction/morphology of the bacterial cells is correct.

14.4.8 If required, subject the culture to additional characterization by biochemical and/or molecular means.

14.5 **Cryopreservation of host bacterial cultures**: Prepare a broth culture of the desired host bacterial species by inoculating with a flamed loop a colony from the agar plate into 9.0 mL of broth and incubate the tube at the required temperature for 18±2 h. Add to this broth culture 1.0 mL of autoclave-sterilized glycerol, shake well and reincubate for 2 h before mixing well and aliquoting into labelled (with indelible ink) cryovials each displaying the source, scientific name, passage number, lot number and date of storage of the test bacterium. Store the vials at -70ºC or below for no longer than 18 months.

14.6 **Host bacterial culture preparation:**

14.6.2 Thaw frozen test culture quickly by holding the vial under running warm water from a tap or by immersing it in a waterbath at 45°C.

14.6.3 To prepare a "Refrigerated Stock Culture", Streak a loopful of the suspension onto a 100 mm diameter LB plates with appropriate antibiotic if necessary (redried to remove any accumulated water on the surface of the agar) to obtain isolated colonies, and incubate the plates at the required temperature for 18±2 h for E. coli and 48±4 h for *Pseudomonas syringae*. Wrap with Parafilm to prevent drying and place at 4±2°C for no longer than 7 days.

14.6.4 Inoculate one colony of the "Refrigerated Stock Culture" into 10 mL of LB with appropriate antibiotic if necessary and incubate for 18±2 h.

14.6.5 Using a Vortex-style device, resuspend the culture for 3-4 s.

14.6.6 Assay the fluid to be nebulized for PFU before and after nebulization by making five 10-fold dilutions (e.g. add 100 µL to 900 µL of PBS). Plate appropriate dilutions in duplicate by pour/spread plating or filtration. If using the Miles & Misra (1938) plating technique, place five 20-µL droplets from each the last three dilutions on a 100 mm plate of TSA with a predried agar surface.

14.6.7 Incubate plates for 18±2 h. Record the PFU and also observe them for any extraneous microbial contamination. The test data would be invalid in case any contamination is detected.

14.7 **Vertebrate cell cultures**

14.7.1 Thaw frozen test culture quickly by holding the vial under running warm water from a tap or by immersing it in a waterbath at 45°C.

14.7.2 Resuspend in 5 mL of EBSS and aliquot suspension into 1 mL volumes for preservation in cryovials under liquid nitrogen. For further details, please refer to ASTM E1053 (2020).

15 B**asic design of the aerobiology chamber:**

15.1 Appendix 1 summarizes the details on the specialized pieces of equipment used in the protocol. The equipment and materials listed are examples only and may be substituted with equivalent items from other sources.

15.2 The aerosol chamber (Figure 1) is an enclosure with a volume of 900.0 ft^3 (25.00 m^3) located inside a clean room with negative pressure and controlled access. The chamber's walls are made out ot wipe-able, solid coroplastic sheeting (https://www.homedepot.com/p/Coroplast-48-in-x-96-in-x-0-157-in-White-Corrugated-Plastic-Sheet-CP4896S/205351385) affixed to a framed structure to represent the walls to maintain an airtight seal. Sealable ports, window and door provide access to the inside of the chamber for maintenance and to place and remove any monitoring devices to be used. The walls should be grounded properly to dissipate any static electricity that may accumulate.

15.3 While the chamber can be used with all major classes of microorganisms at biosafety levels (BSL) 1 and 2, the CDC guidelines (CDC 2020) recommend that the extra safety precautions and operational requirements be in place for work with experimental aerosols of all such microorganisms. Therefore, the aerobiology chamber is house

inside a room with negative pressure and controlled access. This elevates the biosafety containment level of the CREM Co facility to 'BSL-2+'.

15.4 In accordance with the current EPA guidelines (2012), the chamber does not permit any air exchanges; nor does it contain any furniture or fixtures in accordance with EPA 810.2500 study design description. Furniture and fixtures were not placed in the chamber inside of the BSL facility due to biosafety and decontamination concerns over the multiple test dates over a long period.

15.5 The chamber's internal environment is monitored throughout an experiment with a wireless relative humidity (RH)/air temperature sensor/data logger system (e.g., CAS Data Loggers, 8437 Mayfield Rd., Unit 104 Chesterland, OH 44026); www.dataloggerinc.com/) and recorded on cloud for subsequent download and analysis.

15.6 To assess the airborne survival of the test bacteriophages / viruses or to determine the activity of any air treatment technology, the air in the chamber is sampled at the rate of 28.3 L/minute using an externally-placed slit-to-agar air (STA) sampler with a built-in vacuum pump. This programmable device can be set to operate for a minimum air sampling time of 30 seconds to as long as five hours depending to the STA model, and the actual length of sample collection time will be determined by the anticipated load of viable bacteriophage / viruses in the air of the chamber. The air exiting the sampler is discharged directly into a HEPA incorporated in the device or into the BSL-2 facility's HEPA-filtered exhaust system. For the baseline value, the concentration of the test bacteriophage / viruses in the nebulizer fluid should be adjusted to achieve a minimum of 4.2 $\log_{10}$ to a maximum of 5.0 $\log_{10}$ PFU per m^3 at the start of the treatment. Here it should be noted that a recent review indicated that semi-solid impactors are more effective than liquid impingers for air sampling for virus detection (Borges et al., 2021). That is why CREM Co Labs prefers using STA for air sampling.

15.7 Between experiments, the air inside the chamber is replaced with fresh air using a vacuum pump and the exiting air directly discharged into a BSC located in the clean room for a minimum of one hour.

15.8 The Start and Stop times (clock times) will be recorded for the application of the treatment to the air. The official exposure period or contact time begins upon completion of the release of the test substance which should begin after the nebulizer has completed the 10-minute release of the test bacteriophage / virus , five minutes for stabilization of the aerosolized microorganism and the 2 minute pre-treatment air sample is taken.

15.9 Any Spray-formulation can also be placed inside the chamber and activated from the outside or by accessing it with the gloves affixed to the chamber (Figure 1). The labeled use directions will be based upon the test substance application procedure used during testing.

15.10 A magnehelic is affixed to the outside of the chamber to visually indicate on a continuing basis pressure differential between its internal and external atmospheres. Any pressure differential would be regarded as indicative of a breach in the integrity of the chamber resulting in the immediate termination of the test.

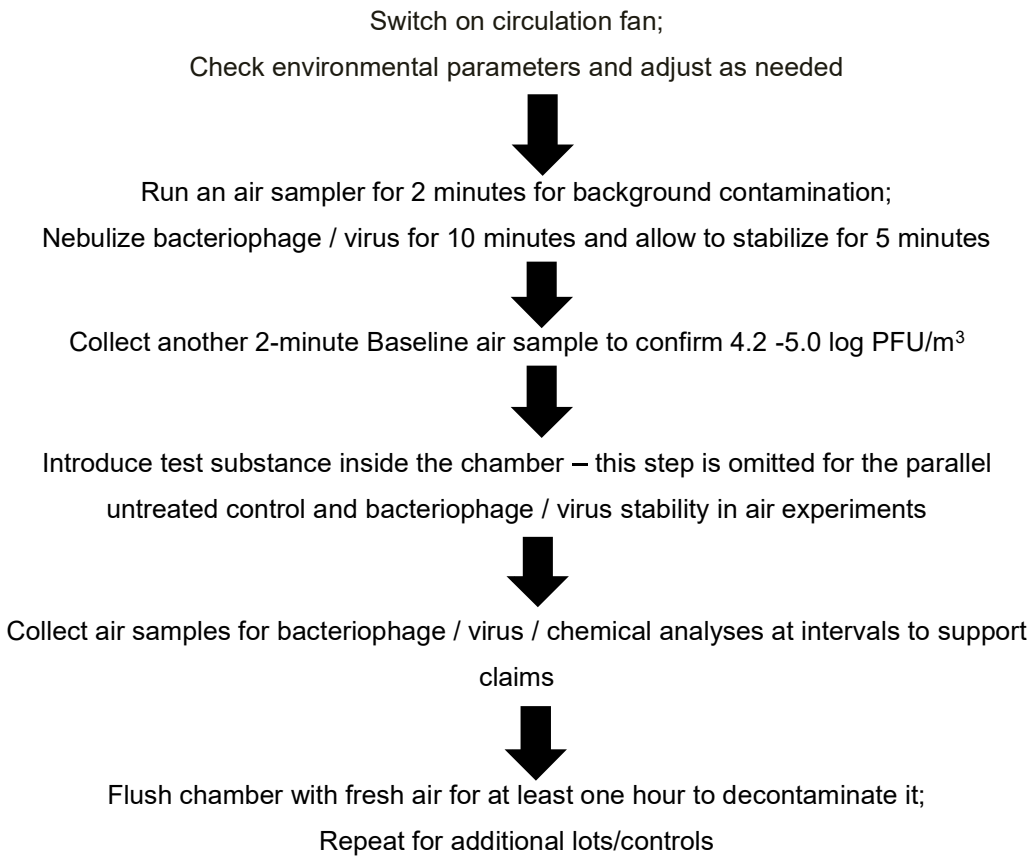
15.11 The exposure period (contact time) may vary with the Test Substance. The same exposure period will be used to evaluate each lot of a Test Substance and controls. The air will be sampled for the same duration and at the same intervals for each lot of a Test Substance and Controls but no fewer than three air samplings per lot per microorganism will be collected.

The air may be sampled for different durations and after different intervals for Test Substance tests and Controls to recover countable PFU on sampling plates and reduce the

detection limit as much as possible. No fewer than three air samplings per lot per microorganism per chamber run will be collected for both Test substance and Controls. Each test lot will be evaluated in three runs of the chamber for each one of the two bacteriophages.

16 **Experimental Design:** A generic sequence of the main steps in the operation of the chamber is given in the Flowchart below.

Flowchart.



17 **Method for Control of Bias**: None

18 **Operation of the aerobiology chamber**

18.1 Actuate the 'muffin' fan (e.g. Cooltron AC Fan, Model FA8038B11T7-51, 80x80x38mm,7Blds,115VAC,50/60Hz,11/9W, 26/31CFM) placed on the floor of the chamber directly underneath the nebulizer inlet pipe (Figure 1) 10±2 minutes prior to nebulization of the bacteriophage / virus suspension. Leave the fan on for the duration of a given test to maintain uniform distribution of the aerosolized particles in the air inside. Between experiments, wipe the outside of the fan with 70% (v/v) ethanol for decontamination.

18.2 Check temperature and RH. Adjust if needed.

18.3 Connect the inlet of the STA sampler to a PVC pipe (ID 5.0 cm) which extends into the center of the chamber (Figure 1).

18.4 Place a 150 mm diam. disposable Petri plate with LB agar or equivalent growth medium inside the sampler. All agar plates used during testing will be equilibrated to room temperature and the surface of the medium dried prior to use. Collect a 2-minute sample using a STA air sampler to measure the background bacteriophage / virus contamination prior to test initiation. After collection, retrieve and incubate the plate alongside the test plates.

18.5 Attach an externally placed six-jet Collison nebulizer (Appendix 1) to the port on the chamber, connect the nebulizer to a compressed air cylinder (Figure 1) and then adjust the air pressure to 25 pounds/square inch (PSI) to nebulize the test microbial suspension for 10 minutes. Allow the bacteriophage / virus to circulate and stabilize for 5 minutes.

18.5.1 The level of bacteriophage / virus in the fluid to be nebulized and the volume nebulized should be previously determined to obtain recovery plates with countable numbers. The sampling times and nebulized fluid should be pre-determined to achieve a minimum of 4.2 $\log_{10}$ to a maximum of 5.0 $\log_{10}$ PFU per m^3 in the baseline.

18.5.2 Any plates with plaques which are too-numerous-to-count (TNTC) will invalidate the corresponding sampling point. For the plate of the first sampling point with no visible growth, use a value of 1.0 PFU to take into account the maximum error in the detection limit of STA air sampler.

18.5.3 Weigh the nebulizer before and after nebulization to determine the volume of fluid nebulized. Each gram of weight is regarded as equal to 1.0 mL of the fluid.

18.5.4 At the end of each experiment, retrieve, decontaminate, clean and autoclave sterilize the pipe and the quick connect attached to the nebulizer.

18.6 Place another 150 mm diameter disposable Petri plate with LB agar or equivalent growth medium (agar surface predried) inside the sampler. Collect a 2-minute air sample to measure the baseline bacteriophage/virus level in the chamber ("Baseline"). This value serves as the parallel, untreated control. After collection, retrieve and incubate the plate alongside the test plates.

18.7 After Baseline sampling, the Test Substance is sprayed into the aerobiology chamber to treat the air. The container of test substance may be released for the desired length of time by pressing its button via the built-in access gloves. (Figure 1). Shake test aerosol spray-can well before use. Hold can upright, press button and spray towards the center of the aerobiology chamber in a sweeping motion. The start and stop times

(clock times) will be recorded for the application of the treatment to the air. The official exposure period or contact time begins upon completion of the release of the test substance.

18.8 Air treatment is omitted when conducting the bacteriophage / Virus--Stability-in-Air Control. This control is conducted to measure the survival and settle rate of the test microorganisms in the chamber over the test period.

18.9 After completing the release of the test substance start sampling the air continuously following Table 1. After incubation, each plate should be divided into 4 sections and the PFU in each section will be counted and recorded.

Table 1: Sampling Duration Example for the Test Substance

Samples	Sampling Duration (minutes)	Four Sections on each recovery plates (minutes)			
		#1	#2	#3	#4
Baseline**	-2 to 0	Section (Sampling time point) minutes			
1	0*-10	0-2.5 (1.25)	2.5-5 (3.75)	5-7.5 (6.25)	7.5-10 (8.75)
2	10-20	10-12.5 (11.25)	12.5-15 (13.75)	15-17.5 (16.25)	17.5-20 (18.75)
3	20-30	20-22.5 (21.25)	22.5-25 (23.75)	25-27.5 (26.25)	27.5-20 (28.75)
4	30-45	30-33.75 (31.875)	33.75-37.5 (35.625)	37.5-41.25 (39.375)	41.25-45 (43.125)
5	45-60	45-48.75 (46.875)	48.75-52.5 (50.625)	52.5-56.25 (54.375)	56.25-60 (58.125)

***** Time 0 start after finishing the application of the test substance

** Sampling for Baseline can be shorter to get a countable PFU on plates if the STA machine has the capability.

18.10 When conducting the Bacteriophage/virus Stability-in-Air Control use Table 2 to for sampling time and duration

Table 2: Sampling Duration for the Test Substance

Samples	Sampling time point (minutes)	Sampling Duration (minutes)
Baseline	0	-2 to 0
1	10	9-11
2	20	19-21

3	30	29-31
4	45	44-46
5	60	59-61

18.11 In order to calculate the time in which the test substance demonstrates an $\geq$3-$\log_{10}$ reduction, PFU/m^3 will be calculated for each sampling time point using the counted PFU on the corresponding section following the formula presented in Appendix 2. The $\log_{10}$ PFU/m^3 will be plotted for each sampling time point for control and efficacy tests. The time in which the test substance demonstrates an $\geq$ 3-$\log_{10}$ reduction in PFU/m^3 will be calculated following procedure explained in Appendix 2.

18.12 Using a towelette soaked in 70% (v/v) ethanol, wipe the outside as well as readily accessible inside surfaces of the sampler after each test. Similarly, decontaminate the outside and inside surfaces of the air sample collection pipe (Figure 1).

19 Chamber Testing Schedule

Appendix 4 illustrates an example testing schedule for 2 lots of test substance on 3 test bacteriophage / virus strains with all required controls.

20 Confirmation of neutralization of active ingredient(s) prior to testing

20.1 Depending on the type and concentration of the active ingredient(s) under test, a neutralizer (with one or more components) is incorporated into the agar medium for air sampling to immediately arrest bactericidal and/or bacteriostatic activity.

20.2 To validate the activity of such a neutralizer to arrest the virucidal activity, introduce the test substance lot with the highest level of the active(s) into the chamber.

20.3 Collect a 30-minute air sample using an STA sampler containing a plate with the recovery agar medium.

20.4 Within 30 minutes after collection of the air sample, spread over the agar plate 100 µL of the test microbial suspension diluted to contain $\leq$100 PFU per plate.

20.5 For controls, (a) use the same procedure to inoculate two unexposed plates containing the neutralizer and two unexposed plates without the neutralizer, and (b) one agar plate from the same lot as sterility control. Incubate plates at 36±1ºC and observe them after 18±2 hours of incubation. Count and record the PFU. The performance criteria are no more than a 20% difference in the numbers of PFU on (a); and (b) should be free from any visible growth.

20.6 The neutralizer is regarded as effective if the number of PFU on the test plates is within 80-120% of the PFU count on the control plate (OECD 2003). Any bacterial or fungal growth on the sterility control plate would invalidate the test.

21 Collection and analysis of test chemical(s) in the air of the chamber

21.1 Collect and analyze air samples from the chamber from a run of the test chemical(s) without the test microorganism for each lot of test substance using a validated chemical analysis method at the same time points and durations used for bacteriological samples collected during treatment. For example, the concentration of Dipropylene Glycol – DPG and Triethylene glycol - TEG in air would be analyzed by Gas Chromatography similar to NIOSH Method # 5523 using a validated method.

22 Incubation

22.1 Incubate all test and control plates at the required temperature (MS2 at 36±1°C and Phi6 at 30±1°C for 18±2 h) and observe them after incubation for bacteriophage / virus PFU.

22.2 Enumerate survivors and calculate bacteriophage / virus survival/reductions.

22.3 See Appendix 2 for calculations.

23 Quality Control

23.1 Sterility Control: One plate of growth media will be incubated alongside the test. All reagents will be evaluated by plating 1.0 mL on growth media and incubated alongside the test. The acceptance criterion for this control is lack of visible growth.

23.2 Viability Control: The growth/neutralizing media will be challenged in duplicate with <100 PFU bacteriophage/virus and incubated alongside the test to confirm the media can support the growth of low numbers.

23.3 Purity Control: A streak plate will be prepared of the test culture and incubated alongside the test to confirm use of a pure culture. The acceptance criterion for this control is the demonstration of a pure culture.

23.4 "Bacteriophage / Virus-Stability-in-Air" Control: This control is conducted as described in Section 20.0 omitting treatment with the test substance (Section 20.9). This control measures the survival and settle rate of the test microorganisms in the chamber over the test period. There are no acceptance criteria for this value. This value is used in the calculation of the reduction performance. This study is conducted at least three times for each bacteriophage / virus.

24 Statistical Analysis

24.1 The data will be subjected to appropriate statistical analyses for the preparation of the final project report. Such analyses will include, at a minimum, calculation of standard deviations (SD).

24.2 Method for Control of Bias: None

25 Study Acceptance Criteria:

25.1 Test Substance Performance Criteria: After correction for aerosol settling and natural biological decay, the test substance must demonstrate ≥99.9% (3 $\log_{10}$) reduction in the viability of each bacteriophage / virus over the parallel untreated control.

25.2 If cytotoxicity is present, the virus/bacteriophage titer should be increased if necessary to demonstrate a ≥3 $\log_{10}$ reduction in PFU/m^3 beyond the cytotoxic level.

25.3 Baseline Acceptance Criteria: The control recovery must demonstrate a minimum of 4.2 $\log_{10}$ to a maximum of 5.0 $\log_{10}$ PFU/m^3 at the start of the treatment for a valid test.

25.4 The maximum allowable contact time to support an air treatment claim should not be longer than 15 minutes for a ≥3 $\log_{10}$ in the level of viability of all species of bacteriophage/viruses to be tested.

26 Control Acceptance Criteria:

26.1 All sterility controls must be free of any visible growth.

26.2 Viability Control must demonstrate growth in all media with <100 PFU/plate.

26.3 Purity Control must demonstrate a pure culture.

26.4 Neutralization Validation: The mean number of PFU on the plate unexposed to the test substance and those on the plate exposed to the test substance must be within 50%.

26.5 Magnehelic readings must indicate no leaks in the chamber during an experiment.
26.6 Air temperature and RH readings must stay within range required for the test.

27 Retesting Guidance

27.1 For tests where the product passes and the mean Baseline value is above 5.0 $\log_{10}$ PFU/m^3, no retesting is necessary. For tests where the product fails and the mean *Baseline* is above 5.0 $\log_{10}$ PFU/m^3, retesting may be conducted. For tests where the product fails and the mean baseline is less than 4.2 $\log_{10}$ PFU/m^3, no retest is required.

28 Protocol Changes

28.1 Protocol changes or revisions, if needed, will be documented including the reason for the change, signed/dated by the Study Director and Sponsor, and described in the study report. SOPs used in the study will be the effective version at the time the study will be conducted. Changes in SOPs not required by the protocol will be documented in the raw data and approved by the Study Director.

29 Study Report:

29.1 The study report will include all elements listed in OCSPP 810.2500, 40 CFR Part 160.185, and EPA Pesticide Registration Notice 2011-3.

30 Study Retention:

30.1 All original raw data for this study will be archived at the Test Facility until study completion including all handwritten raw data for control and test substances (e.g. notebooks, data worksheets, and calculations), protocol amendments/deviations. SOP deviations, study specific correspondence, original signed protocol, and the signed study report (810.2000).
30.2 All facility records for this study will be archived at the Test Facility until study completion including SOP, referenced methods, QA reports, equipment logs, equipment calibration and maintenance logs, reagent preparation and quality control records, personnel training, education, and experience records, etc.
30.3 Following study completion, the Test Facility will keep all raw data and certified copies of facility records for 5 years before transferring them to the RB GLP Archives (Montvale, NJ).

31 Approval Signatures

Name of Sponsor:
Name of Sponsor's authorized representative:
Title:

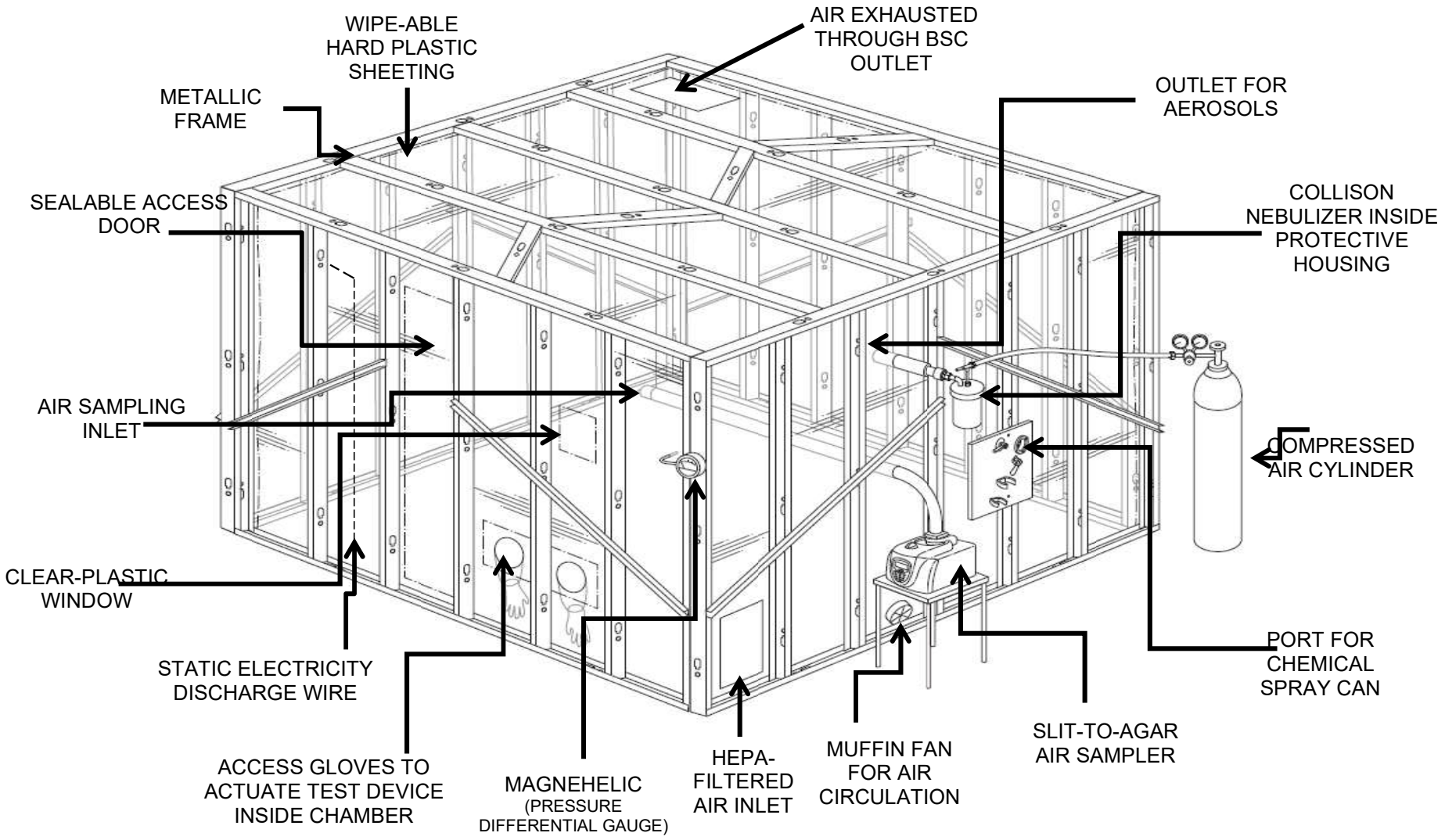
_____

Signature of Sponsor's authorized representative Date

Name of Performing Laboratory:
Name of Study Director:
Title:

Signature of Study Director Date

Figure 1: Aerobiology Test Chamber with Essential Components (900 ft^3 or 25 M^3)



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Appendix 1

Examples of additional specialized pieces of equipment required

Equipment (Cat. #)	Manufacturer	Purpose	Design features/Justification for use	Maintenance/Operation	Illustration
1. Collison six-jet nebulizer (Model: MRE CN24/25)	CH Tech., Westwood, NJ 07675; www.inhalation.org	Generate airborne particles in the respirable range (0.1-5.0 µm in diam.).	Air at a pressure of about 25 psi (172.37 kPa) from a pump or compressed air cylinder is needed for operation. The glass reservoir receives 15 mL of the bacteriophage / virus-soil mixture and it is weighed before and after nebulization to determine the volume aerosolized and may be used to estimate the number of PFU introduced into the chamber air. The liquid to be nebulized contains antifoam to reduce excessive foaming during nebulization. This type of nebulizer is favored in microbial aerobiology due to its versatile and well-characterized nature. The size range of particles generated by it not only are in the respirable range, but the droplet nuclei arising from them can remain suspended in air for periods long enough to study biological decay and/or the impact of physical or chemical agents on the viability of airborne infectious particles.	The entire unit, which is made of metal & glass, can be readily washed and autoclave-sterilized between uses. The nebulizer may be placed inside a shatter-proof plastic housing as extra workplace safety precaution in case of any leakage or breakage of the nebulizer's glass container. This device does not require any periodic recalibration.	
2. RTR-500 series data logger RTR-503L for relative humidity & air temperature (cat. RTR-503L)	CAS Data Loggers, 8437 Mayfield Rd., Unit 104,Chesterland , OH 4 4026 www.dataloggerinc.com	Remotely sense and record relative humidity (RH)/air temperature in the chamber.	RH and air temperature are among the crucial factors affecting microbial survival in air. They may also influence the efficiency of any air decontamination technology being assessed. The recorder is designed to send data wirelessly to a remote computer at an adjustable time interval. A 5-minute interval is used for data capture.	This device requires yearly recalibration by the manufacturer.	

3. Sampler slit-to-agar (STA) sampler.	Pinpoint Scientific Ltd, 1st Floor, North Road, Bridgend Industrial Estate, Bridgend, , CF31 3TP; sales@pinpointscientific.com). Or Particle measuring system 5475 Airport Blvd Boulder, Colorado 80301 USA T: +1 303 443 7100, +1 800 238 1801 W: www.pmeasuring,com	Collect airborne bacteriophage / virus on a timed and event-related basis.	A disposable plastic Petri plate (150 mm diam. X 15 mm in height) with nutrient agar (75 mL) is placed on the sampler's rotating platform to collect aerosols by impingement, and the distance between the bottom surface of the slit and the top surface of the agar is automatically adjusted for optimal particle impingement. The sampler has a built-in vacuum pump to draw in the air to be sampled, and a timer to permit adjustment of air sampling duration from a minimum of 2 minutes to a maximum of 5 hours. At the end of the sampling time, the plate is removed and incubated. The sampler automatically controls the air sample collection rate at 28.3 L/minute. It can also record sampling data for subsequent download to a computer.	Does not require any sterilization between uses but a simple wipe-down with a disinfectant-soaked towelette. Before each use, the air-inlet must be inspected to ensure that the slit is free of any obstructions. The volume of the agar in the Petri plate must be precisely measured to maintain a specific distance between the surface of the agar and the bottom of the slit. The agar surface must also be free of any water drops before placement in the sampler. The sampler has a built-in tubular HEPA filter at the exhaust with a flow-sensor to indicate when the filter needs replacement. This device requires yearly recalibration by the manufacturer.	
4. Volatile gas leak detector and probe	Quantum Instruments; Model BT-45	To detect any leaks in the chamber.	The device could detect either hydrogen gas from a cylinder or glycol introduced into the chamber using a fogger.	Replace battery when stabilization beeps are prolonged past 1 minute.	
5. Muffin fan	Cooltron AC Fan, Model FA8038B11T7-51, 80x80x38mm,7Blds,115VAC,50/60Hz,11/9W, 26/31CFM, Nidec Alpha V, TA300, Model A31022-20, P/N: 933314 3.0 inch/7.62 cm diam.; output 30 CFM	To keep the aerosolized materials uniformly suspended inside the chamber	Relatively small size with sufficient air flow to keep the bacteriophage/virus and the chemicals suspended in air	Requires wiping of external surfaces with 70% (v/v) ethanol	

6. Equipment and accessories to collect samples of the test chemical from the chamber.	Calibrator: check-mate Calibrator, 0.50 to 5 L/min, with NIST Certification, catalog # 375-0550N https://www.skcinc.com/products/chek-mate-calibrator-050-to-5-l-min-1 Pump: Universal 44XR Sample Pump Single Kit, catalog # 224-44XRXD https://www.skcinc.com/products/universal-44xr-sample-pump-single-kit-1 Tubes: Sorbent Tubes, XAD-7 OVS, catalog # 226-57 https://www.skcinc.com/products/sorbent-tubes-xad-7-ovs-1	To collect air-sample for analysis of active(s) .	Air-sampling pump with air-collection tube.	Pump flow-rate needs to be calibrated. For maintenance, follow the instructions provided by the manufacturer.	

Appendix 2

Baseline concentration:

Considering that the sampled air has the same bacteriophage/virus concentration as the air in the chamber, we get the following equation

$$Baseline\ concentration = \frac{PFU\ on\ the\ sampled\ plate}{Volume\ of\ the\ sampled\ air} \quad (1)$$

As the volume of the air sampled relates to the sampling rate and the duration of sampling, Equation (1) gives us the baseline concentration as follows:

$$Baseline\ concentration = \frac{PFU\ on\ the\ sampled\ plate}{\text{sampling rate} \times duration\ of\ sampling\ in\ minutes} \quad (2)$$

The STA sampler samples air at the rate of 0.0283m^3/min. For example, if there are 2516 PFU on the baseline plate with a 2-minutes sample, the baseline concentration can be calculated as follows:

$$Baseline\ concentration = \frac{2516\ PFU}{0.0283\ \frac{m^3}{min} \times 2\ min} = 44452.30\ PFU/m^3$$

which is equal to 4.65 $\log_{10}$ PFU/m^3

Bacteriophage / virus concentration in chamber air

For the *n*th sampling plate, similar to equation (2) we can write:

$$Bacteriophage/Virus\ concentration\ in\ the\ chamber\ air = \frac{PFU\ on\ the\ sampled\ plate}{\text{sampling rate} \times duration\ of\ sampling\ in\ minutes} \quad (3)$$

Removal of the air from the chamber due to collection of each sample dilutes the bacteriophage / virus concentration. The correction factor required to address this is defined as:

$$Dilution\ Correction\ Factor\ for\ nth\ sampled\ plate = \frac{Volume\ of\ the\ chamber}{Volume\ of\ the\ chamber - \text{sampling rate} \times duration\ of\ sampling\ in\ minutes * n} \quad (4)$$

Therefore, the total bacteriophage / virus corresponding to the *n*th sampled plate can be calculated as follows:

$$Corrected\ \text{bacteriophage/virus}\ concentration\ in\ the\ chamber\ air =$$

$$\frac{Volume\ of\ the\ chamber}{Volume\ of\ the\ chamber - \text{sampling rate} \times duration\ of\ sampling\ in\ minutes * n} \times \frac{PFU\ on\ the\ sampled\ plate}{\text{sampling rate} \times duration\ of\ sampling\ in\ minutes}$$

For example, if the third 2-minute sampling plate contains 1 PFU, we can calculate the corrected bacteriophage / virus concentration as follows:

$$Corrected\ bacteriophage/virus\ concentration\ in\ the\ room = \frac{24.34\ m^3}{24.34\ m^3\ - 0.0283\ \frac{m^3}{min} \times\ 2\ min \times 3} \times \frac{1\ PFU}{0.0283\ \frac{m^3}{min} \times\ 2\ min} = 17.79\ \frac{PFU}{m^3}$$

which is equal to 1.2 $\log_{10}$ PFU/m^3

Calculating biological Decay and the Efficacy of a substance

To evaluate the efficacy of a test substance, the rate of biological decay of the challenge bacteriophage/virus species in the air of the chamber is determined first. This is equal to an untreated parallel control. Then, another experiment is conducted (efficacy test) where the test substance is released into the chamber. Since the initial titers of the two experiments may differ in practice, the data for the untreated parallel control is transformed so that its initial titer becomes equal to the initial titer of the efficacy test.

Figure 1 shows PFU recovery data from the untreated parallel control experiment, transformed untreated parallel control and efficacy experiment. Log_{10} reduction at each sampling time is equal to the vertical distance between the transformed untreated parallel control line and the line for the efficacy test.

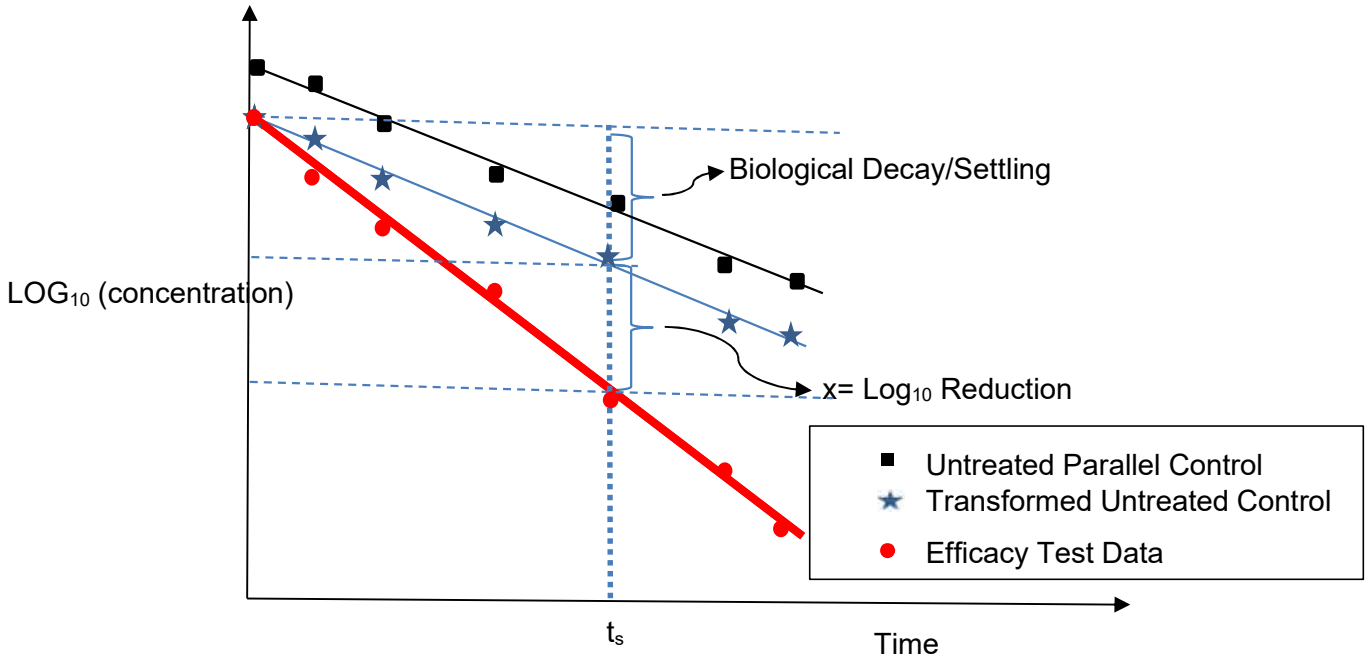

Figure 2: Schematic of log reduction and biological decay

Log_{10} reduction is used to evaluate the substance efficacy, if the $Log_{10}reduction \geq 3$ in a given contact time, the technology is considered to meet the current performance criterion.

Appendix 3: Glossary & Abbreviations

GLOSSARY

Term	Definition
Aerobiology	Study of the behavior of microorganisms, pollen and allergens in air
Air treatment	Removing and/or inactivating potentially harmful microorganisms in air
Nebulizer	Any device capable to turning a powder or liquid into airborne particles
Nebulizer fluid	A suspension of the test microorganism in a soil load and an antifoam
Refrigerated stock	A prepared microbial suspension used to initiate cultures for use in experimentation
Slit-to-agar (STA) air sampler	A device where airborne microbes are drawn through a narrow slit for capture on a nutrient recovery medium by impaction
Soil load	A mixture of one or more organic/inorganic substances added to suspensions of test microbes to simulate the presence of bodily secretions, excretions, or other materials that may shield microbes by interfering with the activity of a microbicidal agent.

Abbreviations

µL	Microliter
BSA	Bovine serum albumin
BSC	Biological safety cabinet
BSL	Biosafety level
DDW	Deionized distilled water
m^3	Cubic meter
h	Hour
HEPA	High-efficiency particle arrestor
ID	Inside diameter
kPa	Kilo-Pascal
m^3	Cubic meter
PBS	Phosphate-buffered saline
PES	Polyethersulfone
PPE	Personal protective equipment
PVC	Polyvinyl chloride
QAU	Quality assurance unit
SD	Standard deviation
STA	Slit-to-agar
TSA	Trypticase soy agar
TSB	Trypticase soy broth
v/v	Volume/volume

Appendix 4

Example of Aerobiological Testing Schedule

Example of daily activities to assess chemical-based formulations for indoor air treatment

Description	Week 1					Week 2					Week 3					Week 4				
	Day					Day					Day					Day				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
MS-2																				
Stability in Air	√	√																		
Neutralization	√																			
Baseline + Lot 1		√	√√																	
Baseline + Lot 2				√√	√															
Phi6																				
Stability in Air							√	√												
Neutralization							√													
Baseline + Lot 1								√	√√											
Baseline + Lot 2										√√	√									

Daily activities for a given lot/microorganism/replicate

Day 1 – AM

- 9:00 –Wear PPE, check the chamber for integrity as well as air temperature and RH, adjust RH if required. Seal the door of the chamber. Prepare suspension for nebulization. Collect the nebulizer fluid for PFU assay.
- 10:00- Nebulize test bacteriophage / virus for control counts (Stability-in-Air) [10-min nebulization + 5-min stabilization + collecting at least four air samples over the next 60-min+ collect the nebulizer fluid for PFU assay.]
- 11:15 – Stability-in-Air (untreated control) assessment complete

Keep the prepared host bacterial culture on ice for use later during the day

- 11:15 – 12:00 – Evacuate the chamber. Dilution and plating of nebulizer fluid samples. Incubation of the culture plates. Observe plates from previous days' experiments and take photographs, clean up and decontaminate lab ware.
- 12:00 – 1:00 PM – Continue Evacuating the chamber
- 12:00 – 1:00 – Scientists take break plus flush the chamber

Day 1 – PM

- 1:00 PM–Wear PPE, check the chamber for integrity as well as air temperature and RH, adjust RH if required. Seal the door of the chamber. Prepare suspension for nebulization. Collect the nebulizer fluid for PFU assay.
- 2:00 PM - Nebulize test bacteriophage / virus
- 2:15 PM – Dispense product
- 2:25 – 3:00 PM – Collect air-sample by STA, remove plate for incubation
- 3:00 PM test completed

3:00 – 5:00 PM – Evacuate the chamber. Dilution and plating of nebulizer fluid samples. Incubation of the culture plates. Decontaminate the labware/waste, prepare culture/supplies for next day and exit BSL-2 facility.